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Sec6/8 Complex Is Recruited to Cell–Cell Contacts and Specifies Transport Vesicle Delivery to the Basal-Lateral Membrane in Epithelial Cells

Kent K. Grindstaff,*‖ Charles Yeaman,*‖ Niroshana Anandasabapathy,* Shu-Chan Hsu,*† Enrique Rodriguez-Boulan,‡ Richard H. Scheller,*† and W. James Nelson*§

*Department of Molecular and Cellular Physiology
†The Howard Hughes Medical Institute
Stanford University School of Medicine
Stanford, California 94305-5345
‡Dyson Vision Research Institute and
Department of Cell Biology and Anatomy
Cornell University Medical College
New York, New York 10021

Summary

In budding yeast, the Sec6/8p complex is essential for generating cell polarity by specifying vesicle delivery to the bud tip. We show that Sec6/8 homologs are components of a cytosolic, ~17S complex in nonpolarized MDCK epithelial cells. Upon initiation of calcium-dependent cell–cell adhesion, ~70% of Sec6/8 is rapidly ($t_{1/2} \approx$ 3–6 hr) recruited to sites of cell–cell contact. In streptolysin-O-permeabilized MDCK cells, Sec8 antibodies inhibit delivery of LDL receptor to the basal-lateral membrane, but not $p75^{NTR}$ to the apical membrane. These results indicate that lateral membrane recruitment of the Sec6/8 complex is a consequence of cell–cell adhesion and is essential for the biogenesis of epithelial cell surface polarity.

Introduction

Generation of structural and functional polarity is important in budding yeast and in tissues and organs of multicellular organisms including neurons and epithelia. A general model for the development of cell polarity in these diverse systems has been proposed (Drubin and Nelson, 1996). In this model, cell polarity is initiated by spatial cues at the cell surface that promote localized assembly of a submembrane cytoskeleton and reorganize secretory pathways along an axis of polarity relative to the positions of these spatial cues. In budding yeast, the bud scar acts as a spatial cue, and a hierarchy of signaling complexes promotes localized assembly of actin and septin cytoskeleton structures (Herskowitz et al., 1995; Pringle et al., 1995; Drubin and Nelson, 1996). Concomitantly, a targeting patch is established that specifies the site for transport vesicle docking leading to bud growth. Genetic analysis has revealed 17 genes whose products are required for exocytosis in yeast (Schekman, 1992). Six of these gene products, Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, and Sec15p, plus a novel gene product, Exo70p, are present in a multiprotein complex that is associated with the plasma membrane at the bud tip (TerBush and Novick, 1995; TerBush et al., 1996). The restricted localization of this complex to the bud tip, in contrast to the uniform distribution of t-SNARE proteins Sso1/2p and Sec9p on the plasma membrane, implies that the Sec6/8p complex is important in specifying vesicle delivery to a restricted site on the plasma membrane (Brennwald et al., 1994; TerBush and Novick, 1995).

In polarized epithelial cells, cell–cell and cell–substrate adhesion initiate the formation of structurally and functionally distinct apical and basal-lateral plasma membrane domains (Vega-Salas et al., 1987; Wang et al., 1990; Wollner et al., 1992). Sorting of apical and basal-lateral proteins into distinct transport vesicles in the *trans*–Golgi network (TGN) is essential for the development of epithelial cell polarity (Matter and Mellman, 1994). However, protein sorting in the TGN is not sufficient to generate cell surface polarity since apical and basal-lateral proteins are sorted into distinct post-Golgi transport vesicles in fibroblasts but have random distributions on the cell surface (Müsch et al., 1996; Yoshimori et al., 1996). Evidence for the involvement of v- and t-SNAREs in plasma membrane delivery of post-Golgi transport vesicles in polarized epithelial cells has been presented (Ikonen et al., 1995; Low et al., 1996). However, it is unclear whether these protein complexes are sufficient to specify vesicle delivery to apical and basal-lateral membranes.

Recently, mammalian homologs of Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and Exo70p have been identified in neurons (Ting et al., 1995; Hazuka et al., 1997; Kee et al., 1997). Similar to yeast, these proteins are present in a large complex consisting of at least seven proteins (Hsu et al., 1996). The association of Sec8 with the plasma membrane in hippocampal neurons suggests that the Sec6/8 complex is involved in vesicle delivery in neurons (Hsu et al., 1996).

We show that the Sec6/8 complex is expressed in Madin-Darby canine kidney (MDCK) epithelial cells. The Sec6/8 complex is rapidly recruited from the cytosol to cell–cell contacts upon initiation of calcium-dependent cell–cell adhesion. As cell polarity develops, the distribution of the Sec6/8 complex becomes restricted to the apical-junctional complex on the lateral membrane domain. In polarized MDCK cells, antibodies to Sec8 specifically inhibit vesicle delivery to the basal-lateral, but not apical membrane. We suggest that the Sec6/8 complex specifies delivery of vesicles containing basal-lateral proteins to sites of cell–cell contact leading to the biogenesis of a new membrane domain.

Results

Sec6 and Sec8 Are Present in a High Molecular Weight Complex in MDCK Cells

In budding yeast and neurons, Sec6 and Sec8 are components of a large, 7.4×10^5 dalton, ~17–19 S protein complex, of which 70%–90% pellets at 100,000 × g (Bowser and Novick, 1991; Hsu et al., 1996; TerBush et al., 1996). Monoclonal antibodies raised against neuronal homologs of Sec6 and Sec8 reacted with proteins

§To whom correspondence should be addressed.
‖These authors contributed equally to this work.

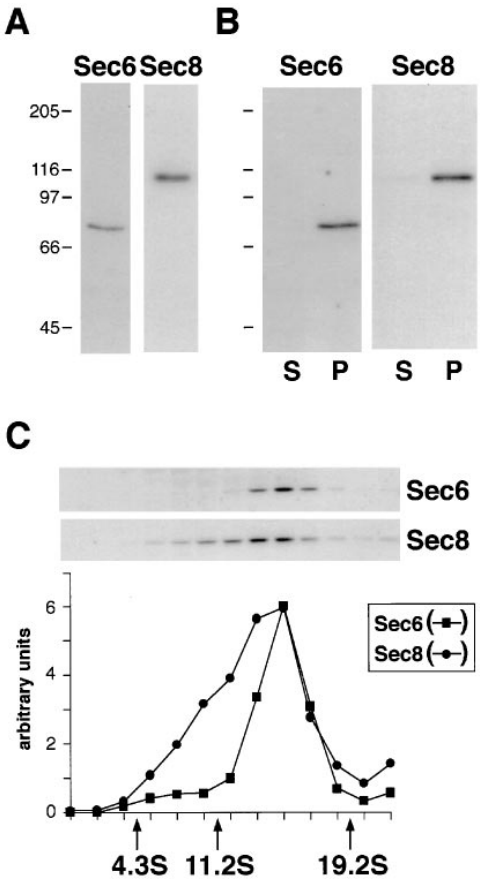


Figure 1. Mammalian Sec6 and Sec8 Are Components of an ~17S Complex in MDCK Cells

(A) Immunodetection of Sec6 and Sec8 in detergent lysates from differentiated MDCK cells. Proteins (10 μg/lane) were separated by SDS-PAGE and electrophoretically transferred to Immobilon P membranes. Membranes were probed with monoclonal antibodies to Sec6 or Sec8.

(B) Immunodetection of Sec6 and Sec8 in high-speed supernatant (S) and pellet (P) fractions. Differentiated MDCK cells were homogenized in isotonic sucrose, and postnuclear supernatant was centrifuged at 100,000 × g for 1 hr. Equivalent volumes of supernatant and pellet fractions were separated by SDS-PAGE and electrophoretically transferred to Immobilon P membranes. Membranes were probed with monoclonal antibodies to Sec6 or Sec8. Protein standards indicated are myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), and egg albumin (45 kDa).

(C) Cosedimentation of Sec6 and Sec8 by velocity gradient centrifugation. Proteins from MDCK cell homogenates were separated in a linear 22.5%–36% (v/v) glycerol gradient. Distributions of Sec6 and Sec8 were determined by SDS-PAGE followed by immunoblotting with specific monoclonal antibodies. Protein levels were quantified using a Molecular Dynamics phosphorimager. Size markers (indicated by the arrows) are bovine serum albumin (4.3S), β-amylase (11.2S), and thyroglobulin (19.2S).

of 8.6 and 11 × 10^4 daltons, respectively, on immunoblots of MDCK cell lysates (Figure 1A); these molecular masses are the same as those of neuronal Sec6 and Sec8 (Ting et al., 1995). Both Sec6 and Sec8 were present primarily in the 100,000 × g pellet fraction from MDCK cell lysates; less than 5% of either protein was detected in the high-speed supernatant (Figure 1B). The size of the MDCK Sec6/8 complex was determined by velocity centrifugation in a linear glycerol gradient and compared to the sedimentation of standard proteins of known S values. MDCK Sec6 and Sec8 cosedimented in fractions with an estimated sedimentation coefficient of ~17S (Figure 1C). A portion of Sec8 also sedimented in an ~11S complex. The identity of this latter complex is unclear at present but may represent a subset of proteins in a partially assembled or disassembled complex. Together, these results demonstrate that MDCK cells express a large protein complex containing Sec6 and Sec8, which has properties similar to those described for this complex in neurons and yeast.

Sec6/8 Complex Is Recruited to the Membrane from a Cytosolic Pool during Development of Epithelial Polarity

Though apical and basal-lateral proteins are sorted into distinct post-Golgi transport vesicles in both nonpolarized and polarized cells (Müsch et al., 1996; Yoshimori et al., 1996), targeted delivery of these transport vesicles to distinct plasma membrane domains in MDCK cells requires cell–cell adhesion (Mays et al., 1995; Grindstaff et al., 1998). Thus, targeted delivery of transport vesicles appears to require the establishment of specific vesicle docking sites at the plasma membrane. To determine whether the Sec6/8 complex displays characteristics consistent with a role in this process, we examined the expression of the complex at different stages during development of MDCK cell polarity. Immunoblotting of postnuclear supernatants from contact-naive and confluent monolayers of MDCK cells showed that similar amounts of Sec6 and Sec8 were present in both nonpolarized and polarized cells (Figure 2A), demonstrating that expression of the Sec6/8 complex was not induced by cell–cell adhesion.

To determine whether the subcellular distribution of the Sec6/8 complex changed during development of epithelial polarity, the fraction of the complex associated with plasma membrane was quantified at different times following initiation of cell–cell contacts. Due to the large size of the Sec6/8 complex, neither differential centrifugation nor flotation in linear sucrose gradients satisfactorily resolved membrane-associated Sec6/8 from cytosolic Sec6/8 (see also Bowser and Novick, 1991). Therefore, we fractionated postnuclear supernatants by centrifugation in a self-forming iodixanol (Opti-Prep) gradient (1.10 g/ml to 1.25 g/ml). Plasma membranes, with a density of ~1.10 g/ml, are recovered at the top of the gradient; cytosolic proteins, with an average density of ~1.25 g/ml, are recovered at the bottom of the gradient. E-cadherin, a plasma membrane protein, was confined to the top two fractions in Opti-Prep gradients of contact-naive and polarized MDCK cells (Figure 2B). In contact-naive cells, >90% of Sec6 and Sec8 migrated to a position near the bottom of the gradient, indicating that the Sec6/8 complex was largely present in a cytosolic pool. In contrast, Opti-Prep separation of polarized MDCK cell extracts showed that ~70% of Sec6 and Sec8 migrated near the top of the gradient, in the same fractions that contained the plasma membrane protein E-cadherin. Therefore, in fully polarized MDCK cell monolayers, the Sec6/8 complex is primarily associated with the plasma membrane.

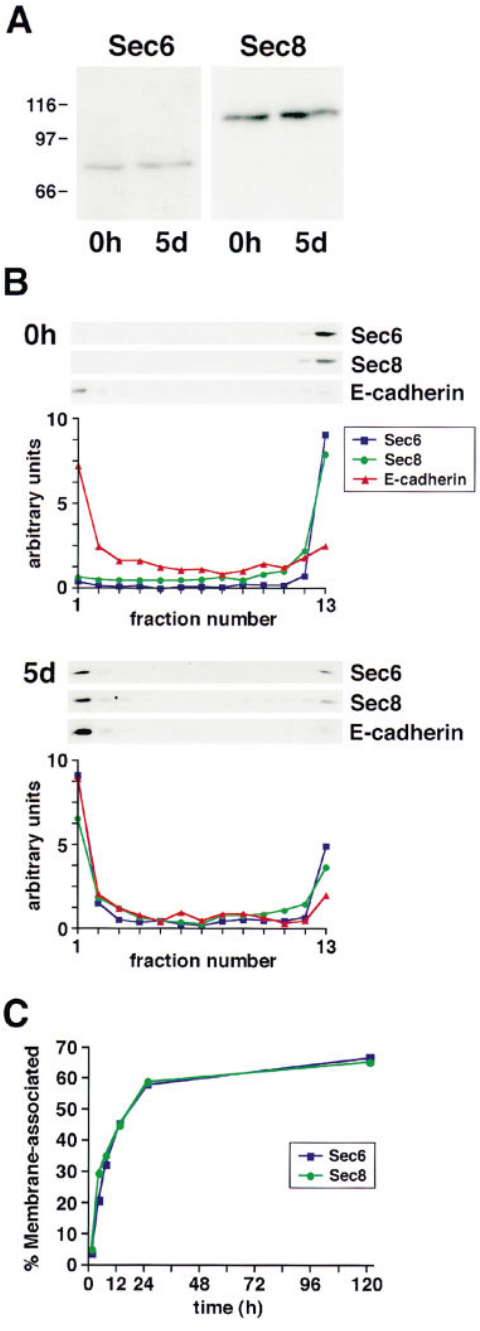


Figure 2. The Sec6/8 Complex Is Recruited to the Plasma Membrane during Development of Epithelial Polarity

(A) Immunodetection of Sec6 and Sec8 in homogenates from contact-naive (0 hr) and differentiated (5-day) MDCK cells. Proteins (25 μg/lane) were separated by SDS-PAGE and electrophoretically transferred to Immobilon P membranes. Membranes were probed with monoclonal antibodies to Sec6 or Sec8. Protein standards indicated are the same as in the legend for Figure 1.

(B) MDCK cell homogenates prepared from contact-naive (0 hr) and differentiated (5-day) cultures were were mixed with iodixanol (30% v/v) and centrifuged at 350,000 × g for 1 hr. The presence of Sec6, Sec8, and E-cadherin in each gradient fraction was assayed by SDS-PAGE followed by immunoblotting with specific monoclonal antibodies. Protein levels were quantified using a Molecular Dynamics phosphorimager.

(C) Homogenates of MDCK cells were prepared 0, 3, 6, 12, 24, and 120 hr following establishment of cell–cell contacts and fractionated as in (B). Membrane-associated material was defined as material recovered in the top two fractions.

The kinetics of recruitment of the Sec6/8 complex to the plasma membrane were assessed by measuring the amounts of Sec6 and Sec8 recovered in the top two fractions from Opti-Prep gradients of homogenates of MDCK cells that had formed cell–cell contacts for 3, 6, 12, or 24 hr. This analysis revealed that the Sec6/8 complex is rapidly recruited to the plasma membrane following the onset of cell–cell contact with a $t_{1/2} \approx 3$–6 hr (Figure 2C).

In order to gain further insight into the biochemical nature of the association of the Sec6/8 complex with the plasma membrane at different stages during the development of cell polarity, membrane fractions were isolated from cells that had formed cell–cell contacts for 6 hr or 5 days. Membranes were extracted with various chaotropic agents and fractionated by centrifugation in Opti-Prep gradients to assess whether the Sec6/8 complex remained in the plasma membrane fraction. Buffers commonly used to extract peripheral membrane proteins extracted relatively little Sec6/8 complex from plasma membrane isolated from early contacting cells or from fully polarized monolayers. Sec6/8 complex was only partially released from membranes following extraction with buffer containing either 4 M urea (76%–87% membrane-bound), or 0.5 M carbonate, pH 11 (70%–80% membrane-bound), or 1 M KCl (~70% membrane-bound). Solubilization of membranes in buffer containing 1% Triton X-100 caused >90% of the Sec6/8 complex to migrate to a position near the bottom of the Opti-Prep gradient, indicative of a protein complex no longer associated with membrane lipids. These extraction conditions did not reveal significant differences in the strength of Sec6/8 association with plasma membranes isolated from cells at different stages of polarity development. Taken together, these data indicate that cell–cell adhesion initiates the rapid recruitment of Sec6/8 from the cytosol to the plasma membrane, where the complex becomes stably associated through tight nonionic interactions.

Sec6/8 Complex Associates with Adhesion Protein Complexes at Sites of Early Cell–Cell Contacts

Since Sec6 and Sec8 are recruited from the cytosol to the plasma membrane following the onset of cell–cell adhesion, we examined the possibility that the complex becomes localized to sites of cell–cell contacts. Figure 3 shows low-density cultures of MDCK cells that had just begun to establish cell–cell contacts. In methanol-fixed cultures, Sec6, the cell adhesion protein E-cadherin, and the tight junction–associated protein ZO-1 exhibited a predominantly diffuse staining pattern. However, cells that had formed cell–cell contacts also exhibited Sec6 staining that was clearly localized to the plasma membrane within the boundary of each cell–cell contact. Both E-cadherin (arrows in Figures 3A and 3B) and ZO-1 (arrows in Figures 3E and 3F) coaccumulated at sites of cell–cell contact with Sec6. Staining for all of these proteins is weak and diffuse in the cytosol or plasma

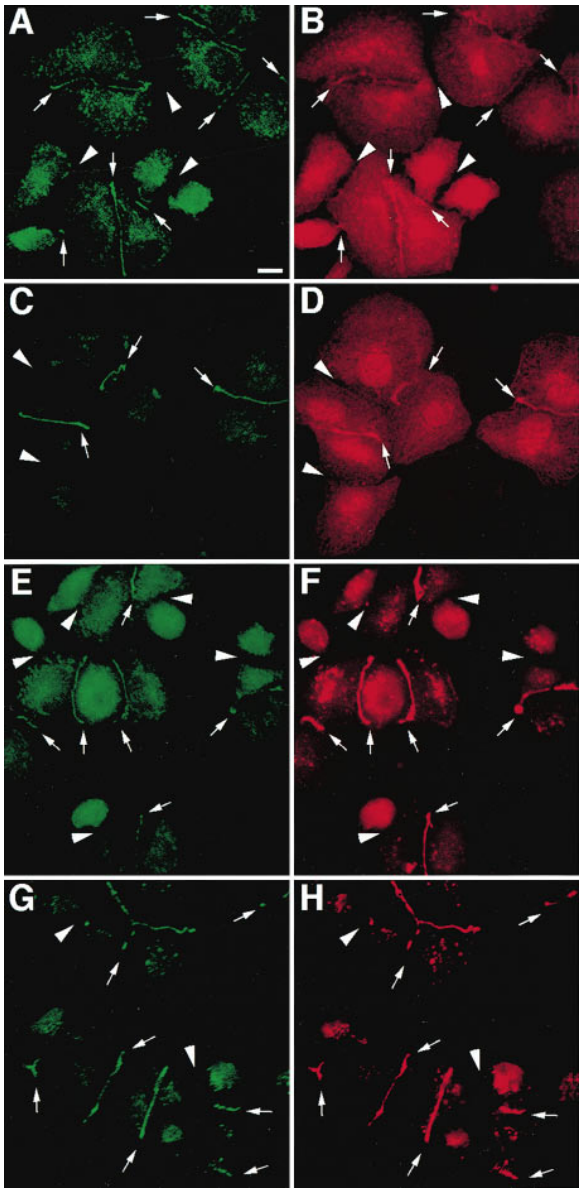


Figure 3. Colocalization of Sec6 with E-cadherin and ZO-1 at Early Cell–Cell Contacts

Low-density cultures of MDCK cells were fixed/permeabilized in methanol (A, B, E, and F) or extracted with 1% Triton X-100 prior to methanol fixation (C, D, G, and H). Sec 6 distribution (A, C, E, and G) was compared to that of either E-cadherin (B and D) or ZO-1 (F and H). Anti-Sec6 monoclonal antibody was visualized with a fluorescein-labeled donkey anti-mouse antibody. Antibodies to ZO-1 and E-cadherin were visualized with rhodamine-labeled donkey anti-rat or anti-rabbit antibodies, respectively. Arrows indicate cell–cell contact sites. Arrowheads indicate noncontacting sites on the plasma membrane. Bar = 10 μm.

membrane outside of cell–cell contact sites (see arrowheads in Figures 3A, 3B, 3E, and 3F). Note that in single cells in contact with the collagen substrate, the Sec6 and ZO-1 staining was restricted to the cytosol. In cells extracted with Triton X-100 prior to fixation with methanol, Sec6 and ZO-1 staining was primarily restricted to sites of cell–cell contacts; relatively little staining was detected elsewhere, indicating that these proteins were largely extracted from the cytosol and areas of the plasma membrane outside of cell–cell contacts (Figures 3G and 3H). Note that while the distribution of Sec6 and E-cadherin is very similar in early contacts, slight differences are apparent (Figures 3C and 3D). Staining patterns of Sec8 in early contacts were indistinguishable from those of Sec6 (data not shown). These results indicate that the Sec6/8 complex is recruited to the plasma membrane at sites of cell–cell contact, and a portion of the complex is stabilized in a Triton X-100-insoluble residue that also contains components of junctional complexes.

Sec6/8 Complex Is Restricted to the Lateral Membrane Domain of Polarized MDCK Cells

In budding yeast, the Sec6/8 protein complex has a highly polarized distribution at the bud tip during bud formation (TerBush and Novick, 1995). In polarized MDCK cells, Sec6 and Sec8 were distributed in a circumferential ring around each cell in association with the lateral plasma membrane domain (Figure 4). Staining of Sec6 and Sec8 was not detected on either the basal or apical membranes. The Sec6/8 distribution in these polarized cells no longer overlapped extensively with that of E-cadherin, as had been observed in cells during initial stages of cell–cell adhesion (Figure 3). While E-cadherin was broadly distributed along the length of the lateral membrane, both Sec6 and Sec8 were restricted to the apex of the lateral membrane in a distribution similar to that of the tight junction–associated protein ZO-1 (Figure 4).

Polarized distribution of the Sec6/8 complex at the apex of the lateral membrane domain was dependent on maintenance of calcium-dependent cell–cell adhesion. Treatment of cells with the calcium chelator EGTA resulted in the rapid (1–4 hr) dissolution of the apical junctional complex (as monitored by ZO-1 localization, Figures 5A and 5C) and redistribution of Sec6 to the cytoplasm (Figures 5B and 5D). Membrane dissociation of Sec6 was reversible. Within 2.5 hr following readdition of calcium to the culture medium, Sec6 was rerecruited to the plasma membrane (Figure 5F) and became restricted to the apex of the lateral membrane within 7.5 hr (Figure 5H). An identical pattern of redistribution was observed for Sec8 (data not shown). These kinetics were similar to those observed during relocation of ZO-1 to the tight junction following calcium add-back (Figures 5E and 5G). Taken together, these data indicate that calcium-dependent cell–cell adhesion provides a signal for initiating the localized recruitment of Sec6/8 to sites of cell–cell contact on the plasma membrane (Figure 3), but as polarity develops the complex acquires a more restricted distribution at the apex of the lateral membrane (Figure 4).

Delivery of Post-Golgi Transport Vesicles to the Basal-Lateral Membrane Requires the Sec6/8 Complex

In budding yeast, mutations in genes encoding components of the Sec6/8p complex cause accumulation of

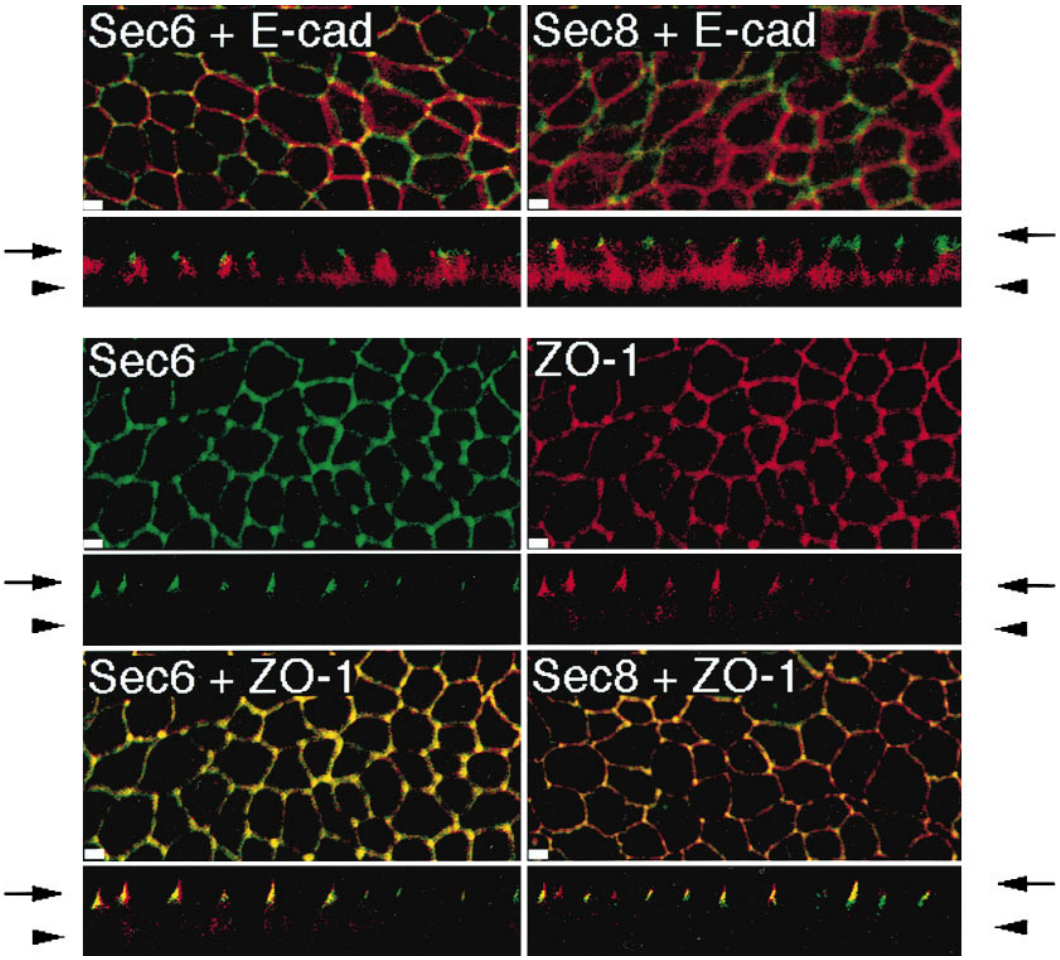


Figure 4. The Sec6/8 Complex Is Restricted to the Lateral Membrane of Polarized MDCK Cells

Polarized MDCK cells cultured on polycarbonate filters were fixed/permeabilized in methanol. Sec6 and Sec8 distributions were compared to that of either E-cadherin or ZO-1. Anti-Sec6 and Sec8 monoclonal antibodies were visualized with fluorescein-labeled donkey anti-mouse antibodies. Antibodies to E-cadherin and ZO-1 were visualized with rhodamine-labeled donkey anti-rabbit or anti-rat antibodies, respectively. Confocal images in the upper panels were acquired along the x–y axis (en face view) of the cell monolayer. The x-z views, in the lower panels, were constructed by averaging sections over a line at each z positon in 0.2 μm steps. Arrows indicate the position of the apical plasma membrane; arrowheads indicate the position of the filter. Bar = 10 μm.

transport vesicles in the cytoplasm (Salminen and Novick, 1989; Finger and Novick, 1997). These data indicate that the Sec6/8p complex is involved in specifying docking of post-Golgi transport vesicles with the plasma membrane at the bud tip. Given the restricted distribution of Sec6/8 homologs to the lateral membrane domain of polarized MDCK cells, we sought to test whether the Sec6/8 complex regulates transport vesicle delivery to specific membrane domains.

To synchronize vesicle transport between the Golgi complex and plasma membrane, MDCK cells were infected with recombinant adenoviruses that encode either an apical membrane protein ($p75^{NTR}$), or a basal-lateral membrane protein (LDL receptor; LDLR). Cells were incubated at 20°C for 120 min in the presence of ^{35}S-sulfate to accumulate and label newly synthesized membrane proteins simultaneously in a late Golgi compartment. Cells were permeabilized with streptolysin-O (SLO) and incubated under different conditions (see the Experimental Procedures). Intact cells, without SLO treatment, were used to determine the total amount of each protein delivered to the cell surface during a 1 hr chase at 37°C. Protein delivery to the apical or basal-lateral membrane was detected by domain-specific cell surface biotinylation. Representative samples from one independent experiment are presented (Figure 6A); data from one experiment performed in triplicate are shown as a histogram (Figure 6B); and data from three independent experiments, each performed in triplicate, are tabulated in Figure 6C.

In intact MDCK cells, approximately 20% of ^{35}S-labeled $p75^{NTR}$ and LDLR were biotinylated and recovered in avidin precipitates following a 1 hr chase at 37°C (Figure 6B). However, we determined that 75%–80% of the ^{35}S-labeled $p75^{NTR}$ and LDLR were delivered to the plasma membrane during this chase (data not shown). The numbers reported in Figure 6 therefore represent the fraction of total ^{35}S-labeled protein recovered in avidin precipitates and have not been corrected for efficiency of biotinylation and avidin precipitation. In cells incubated at

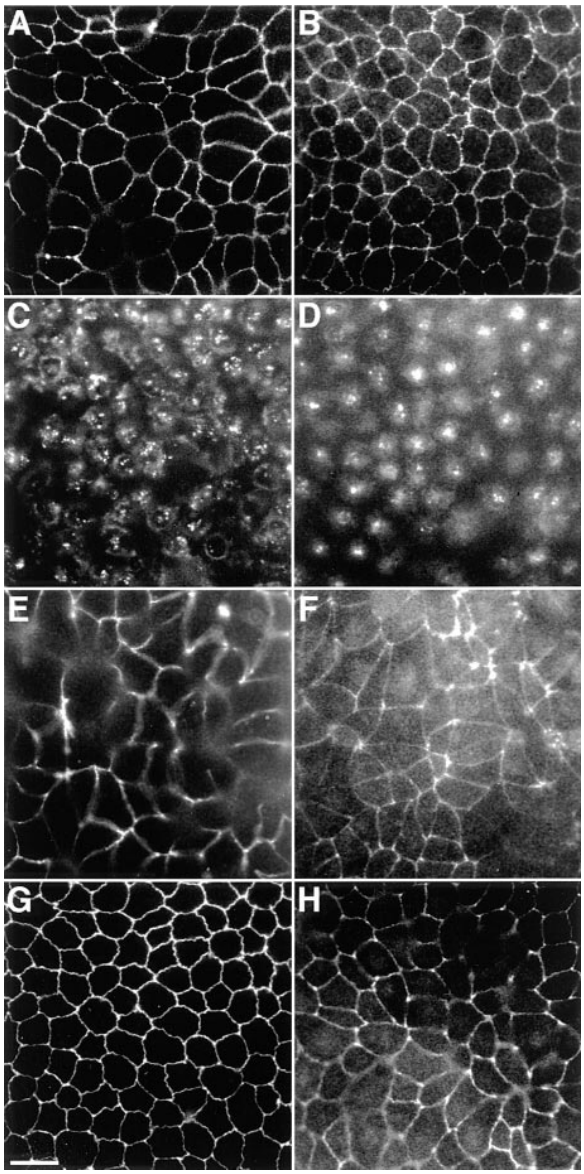


Figure 5. Disruption of Calcium-Dependent Cell–Cell Contacts Causes Dissociation of the Sec6/8 Complex from the Plasma Membrane

Polarized MDCK cells cultured on polycarbonate filters were incubated in DMEM containing 1.8 mM calcium (A–B) or 5 μM calcium plus 2 mM EGTA (C–D) for 4 hr at 37°C. In (E)–(H), cells were cultured in DMEM containing 5 μM calcium plus 2 mM EGTA for 4 hr at 37°C, then shifted into medium containing 1.8 mM calcium and incubated for an additional 2.5 hr (E and F) or 7.5 hr (G and H) at 37°C. Parallel cultures were fixed, permeabilized, and incubated with antibodies to either ZO-1 (A, C, E, and G) or Sec6 (B, D, F, and H). Antibodies were visualized with fluorescein-labeled donkey anti-rat or anti-mouse antibodies. Bar = 20 μm.

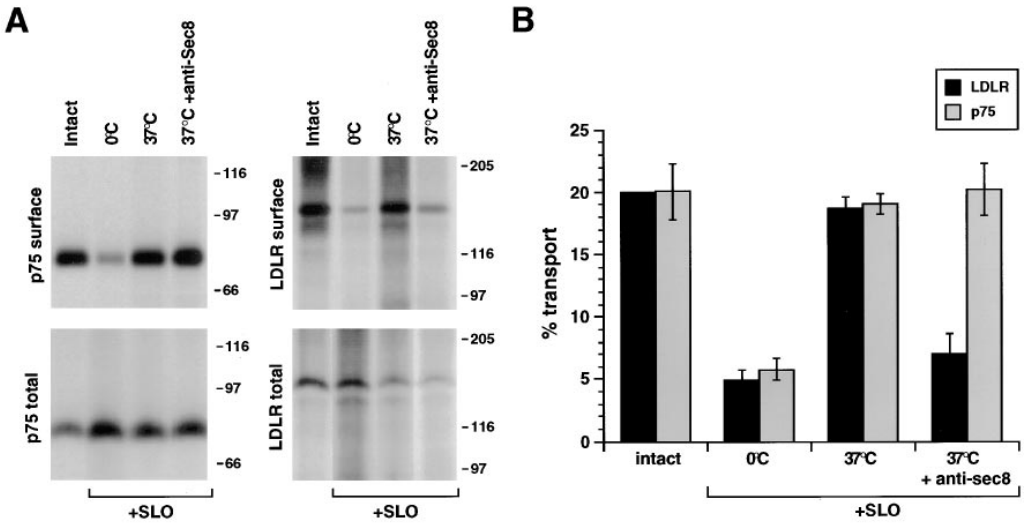
0°C following SLO permeabilization, <5% of $p75^{NTR}$ or LDLR were delivered to the plasma membrane (Figure 6B). Incubation of permeabilized cells at 37°C in the presence of exogenous cytosol and energy reconstituted delivery of $p75^{NTR}$ and LDLR to ~90% of that observed in intact cells. Significantly, addition of Sec8 antibodies, but not nonspecific mouse immunoglobulins (data not shown), to the transport assay inhibited delivery of LDLR to the basal-lateral membrane by approximately 2.5-fold, whereas delivery of $p75^{NTR}$ to the apical membrane was unaffected. These results show that Sec8 antibodies inhibit delivery of vesicles to the basal-lateral, but not apical membrane domain.

Discussion

Although genetic analysis has shown that the Sec6/8p complex is required for polarized secretion in budding yeast (Novick et al., 1981), we present biochemical evidence that homologs of this complex are involved in this process in mammalian cells. Furthermore, we show that the Sec6/8 complex has a restricted distribution on the lateral membrane domain of epithelial cells and provide insight into how it becomes localized there.

Previous studies on vesicle docking and fusion in mammalian systems have focused on the role of interactions between vesicle proteins termed v-SNAREs (i.e., VAMP/synaptobrevin) and target membrane proteins termed t-SNAREs (i.e., syntaxin and SNAP-25). Studies with bacterial toxins known to cleave specifically either VAMP, SNAP-25, or syntaxin have shown that these proteins are important in mediating vesicle fusion with the presynaptic membrane in neurons and the basal-lateral membrane of MDCK epithelial cells (Ikonen et al., 1995; Scheller, 1995). In budding yeast, the plasma membrane homologs of syntaxin (Sso1/2p) and SNAP-25 (Sec9p) are not restricted to the tip of the forming bud, where transport vesicles fuse with the plasma membrane, but are localized over the whole cell surface (Brennwald et al., 1994). Therefore, v- and t-SNARE proteins define a set of membranes that have the capacity to bind to each other and thereby promote vesicle fusion with a target membrane. However, SNARE proteins alone are clearly not sufficient to specify vesicle docking and fusion sites. In contrast, the Sec6/8p complex is restricted to the bud tip and may provide an additional component to the vesicle docking machinery that specifies a subdomain of the plasma membrane for efficient vesicle delivery. Our results demonstrate directly that endogenous Sec6/8 complex specifies transport vesicle delivery between the TGN and the basal-lateral membrane of polarized epithelial cells.

In SLO-permeabilized MDCK cells, antibodies to Sec8 significantly reduced delivery of LDL receptor-containing vesicles to the basal-lateral membrane. We speculate that antibodies bound to the Sec6/8 complex inhibited delivery of LDL receptor-containing vesicles by sterically hindering vesicle docking. Note that due to the small size of the pores created by SLO and resistance of the Sec6/8 complex to extraction from the plasma membrane, we were unable to deplete the complex from cells or restore it exogenously. That basal-lateral vesicle delivery is reduced, but not completely inhibited by Sec8 antibodies indicates that a portion of the Sec6/8 complex may not be accessible to these antibodies. Alternatively, the level of protein delivery observed in the presence of Sec8 antibodies might reflect a baseline level of vesicle docking that is mediated by v- and t-SNAREs alone without the Sec6/8 complex. If the Sec6/8 complex specifies a subdomain of the plasma membrane



C

	p75			LDLR		
	expt 1	expt 2	expt 3	expt 1	expt 2	expt 3
intact cells, 37°C	20.0 ± 2.2	16.9 ± 0.3	22.9 ± 0.7	20	19.8 ± 0.9	30.8 ± 0.9
permeabilized, 0°C	5.8 ± 0.9	7.3 ± 0.7	8.1 ± 0.5	5.0 ± 0.8	7.2 ± 1.3	4.6 ± 1.1
permeabilized, 37°C	19.1 ± 0.8	14.6 ± 0.2	20.0 ± 0.6	18.7 ± 1.0	22.4 ± 0.6	26.6 ± 2.2
permeabilized, 37°C + Sec8 antibodies	20.2 ± 2.1	16.7 ± 1.7	21.6 ± 2.9	7.0 ± 1.6	13.0 ± 0.7	12.2 ± 1.0

Figure 6. Anti-Sec8 Antibodies Inhibit Basal-Lateral Transport of LDL Receptor but Not Apical Transport of $p75^{NTR}$

Cell surface delivery of a basal-lateral membrane protein (LDLR) and apical membrane protein ($p75^{NTR}$) in intact or SLO-permeabilized MDCK cells was assayed as described in the Experimental Procedures. For reconstitution of transport in SLO-permeabilized cells, buffer containing an ATP-regenerating system and exogenous cytosol was added; where indicated, anti-Sec8 monoclonal antibodies were added to the transport assay. Representative samples from one independent experiment are shown in (A); data from one experiment performed in triplicate are shown as a histogram in (B); data from three independent experiments, each performed in triplicate, are tabulated in (C).

for efficient vesicle docking, we anticipate that basal-lateral proteins would be delivered less efficiently to the plasma membrane in contact-naive MDCK cells because the Sec6/8 complex is not membrane-bound. Similarly, when the Sec6/8 complex is recruited to the plasma membrane to sites of cell–cell contact, we would anticipate an increased efficiency of delivery of basal-lateral proteins. These predictions are supported by previously published results. Detailed studies of the trafficking of newly synthesized Desmoglein I, a basal-lateral membrane protein, showed than <10% was transported to the plasma membrane of contact-naive MDCK cells during a 1 hr chase (Pasdar and Nelson, 1989). However, upon initiation of cell–cell contacts, Desmoglein I was rapidly ($t_{1/2} \sim 30$ min) and efficiently (>90%) delivered to the plasma membrane (Pasdar and Nelson, 1989).

Our results establish a link between cadherin-mediated cell–cell adhesion, protein delivery to the basal-lateral membrane, and the spatial organization of the Sec6/8 complex. In single MDCK cells in contact with the substratum, Sec6 and Sec8 are diffusely distributed in the cytosol but are rapidly recruited ($t_{1/2} \approx$ 3–6 hr) to the plasma membrane to sites of cell–cell contact after the induction of calcium-dependent cell adhesion. In early contacting cells, the distribution of the Sec6/8 complex extends along the length of each cell–cell contact but abruptly terminates at the boundary of these contacts. The kinetics of Sec6/8 complex recruitment to the plasma membrane are very similar to the kinetics of establishing a direct delivery pathway for transport vesicles between the TGN and the basal-lateral membrane (Mays et al., 1995; Grindstaff et al., 1998). Therefore, a specific basal-lateral transport pathway in polarized epithelial cells could be established rapidly by recruitment of the Sec6/8 complex to the membrane following a cadherin-mediated spatial cue.

In polarized MDCK cells, the Sec6/8 complex is restricted to the apex of the lateral membrane and no longer extends along the length of each cell–cell contact. We note, however, that this distribution of the Sec6/8 complex, though distinct from E-cadherin, nonetheless is dependent upon calcium-dependent cell–cell adhesion. EGTA treatment of cells disrupts E-cadherin mediated cell–cell contacts and results in dissociation of Sec6/8 from the plasma membrane. Following readdition of calcium to the culture medium, the Sec6/8 complex is rerecruited to the plasma membrane and relocalizes to the apical junctional complex with kinetics similar to those observed for ZO-1. While the spatial organization of the Sec6/8 complex changes during the development of cell polarity, it is unclear how this redistribution is established. The complex is largely resistant to extraction with buffers containing urea, carbonate, or KCl, suggesting that membrane-associated Sec6/8 is tightly

bound, as indicated by previous work in neurons (Hsu et al., 1996). This tight association with the plasma membrane is observed within 6 hr of establishing cell–cell contacts, before the complex becomes restricted to apical junctional complex. Therefore, redistribution of the Sec6/8 complex to the apex of the lateral membrane domain does not correlate with increased strength of association with plasma membrane.

Since Sec8 antibodies significantly inhibit basal-lateral protein delivery, the restricted distribution of the complex at the apex of the lateral membrane indicates that the apical junctional complex is a site for transport vesicle delivery. This possibility is not so unlikely in light of previous studies. Early studies of protein trafficking in MDCK cells demonstrated that amino-peptidase N is delivered to the apical junctional complex (Louvard, 1980). Furthermore, rab8 and rab13, small molecular weight GTPases, are localized to the tight junction and, like the Sec6/8 complex, rab13 is recruited to the plasma membrane from a cytosolic pool during development of epithelial polarity (Huber et al., 1993; Zahraoui et al., 1994). Rab8 and rab13 belong to a subfamily of GTPases that exhibits close sequence similarity to Sec4p, a GTPase required for vesicle delivery to the plasma membrane in yeast (Goud et al., 1988). Similarly, rab3B, a homolog of a small molecular weight GTPase (rab3A) involved in synaptic vesicle docking and fusion, is localized to the tight junction in polarized epithelial cells (Weber et al., 1994). In addition, antibodies to rab-GDI specifically inhibit delivery of TGN-derived transport vesicles to the basal-lateral but not apical membrane (Ikonen et al., 1995).

The results presented here can be put into the context of a general model that has been proposed for the development of cell polarity (Drubin and Nelson, 1996). E-cadherin-mediated cell–cell adhesion provides an initial spatial cue that generates asymmetry on the cell surface by distinguishing this region of the plasma membrane from noncontacting regions. This spatial cue is reinforced by the localized assembly of the actin cytoskeleton (Adams et al., 1996), which serves to retain specific membrane proteins at cell-cell contact sites (Nelson and Veshnock, 1987). Recruitment of the Sec6/8 complex from the cytosol to these contact sites, in combination with specific t-SNAREs (Low et al., 1996), establishes a targeting patch on the plasma membrane for delivery of a subset of constitutively sorted post-Golgi transport vesicles carrying basal-lateral proteins. Newly synthesized proteins delivered to these targeting patches generate further structural and functional asymmetry in the plasma membrane, thereby giving rise to a new membrane domain.

Experimental Procedures

Antibodies

Mouse monoclonal antibodies against Sec6 and Sec8 have been described previously (Hsu et al., 1996; Kee et al., 1997). Rabbit polyclonal antibody raised against the cytoplasmic domain of mouse E-cadherin (E2) has been described previously (Mays et al., 1995). Rabbit polyclonal antibody raised against the extracellular domain of mouse E-cadherin (UVO) was kindly provided by Dr. Rolf Kemler (Vestweber and Kemler, 1984). Rat monoclonal antibody against ZO-1 (R40.76) was kindly provided by Dr. Daniel A. Goodenough (Anderson et al., 1988).

Cell Culture Methodology

Madin-Darby canine kidney (MDCK) clone II cells were maintained as previously described (Mays et al., 1995). Confluent monolayers of "contact-naive" MDCK cells were generated by passaging cells at low density (2×10^6 cells/150 mm diameter dish) on consecutive days. Following trypsinization, cells were resuspended in Dulbecco's modified Eagle's media (DMEM) containing 5 μM Ca^{2+}, supplemented with 10% FBS that had been dialyzed against PBS (without Ca^{2+}). Cells were plated in low Ca^{2+} medium at low density ($\sim 5 \times 10^4$ cells/35 mm dish) on collagen-coated coverslips for immunofluorescent staining, or at confluent density ($\sim 2 \times 10^7$ cells/150 mm dish) for biochemical studies.

Gel Electrophoresis and Immunoblotting

Protein samples were separated in 7.5% SDS polyacrylamide gels (Laemmli, 1970). Proteins were electrophoretically transfered from gels to Immobilon PVDF membrane (Millipore Corp., Bedford, MA). Blots were blocked in BLOTTO (5% w/v nonfat dry milk, 0.1% sodium azide in 150 mM NaCl, 25 mM HEPES [pH 7.4] [HBS]) overnight at 4°C. Anti-Sec6 monoclonal antibody (10D11), anti-Sec8 monoclonal antibodies (8F12, 14G1, and 15E2), or anti-E-cadherin polyclonal antisera (E2) were incubated with blots at room temperature for 1 hr. After three washes, 10 min each, in HBS containing 0.1–1% Triton X-100, the blots were incubated with ^{125}I-labeled goat anti-mouse or goat anti-rabbit secondary antibody (ICN, Costa Mesa, CA) for 1 hr at room temperature. Blots were washed as above and exposed for autoradiography. The amount of labeled protein was determined directly using a Phosphorimager (Model 820; Molecular Dynamics, Sunnyvale, CA).

Glycerol Gradient Analysis

Contact-naive MDCK cells were homogenized in isotonic sucrose buffer (20 mM HEPES-KOH [pH 8.0], 90 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM PMSF, and 10 μg/ml each of pepstatin A, leupeptin, and antipain) by repeated passage through a ball bearing homogenizer (Varian Physics, Stanford University). The postnuclear supernatant was centrifuged at 15,000 $\times$ g for 10 min. The resulting supernatant was fractionated in a linear 22.5%–36% (v/v) glycerol gradient by centrifugation at 91,000 $\times$ g for 16 hr as described previously (Ting et al., 1995). Fractions (90 μl) from 1.2 ml gradients were collected. Proteins in each fraction were separated by SDS-PAGE and transferred to Immobilon P membranes for immunoblotting with monoclonal antibodies specific for either Sec6 or Sec8. In parallel, glycerol gradients were centrifuged containing globular protein standards with known sedimentation coefficients: bovine serum albumin (4.3S), β-amylase (11.2S), and thyroglobulin (19.2S).

Iodixanol Gradient Analysis

MDCK cells were homogenized in isotonic sucrose buffer by repeated passage through a ball bearing homogenizer. The postnuclear supernatant was combined with an equal volume of Opti-Prep (60% iodixanol, Nycomed, Oslo, Norway). Samples were centrifuged at 353,000 $\times$ g for 1 hr at 4°C in a Beckman VTi65 rotor. Under these conditions, iodixanol forms a steep density gradient ranging from approximately 1.10 g/ml to 1.25 g/ml (Nycomed Centrifugation Technical Bulletin VII: Gradient Formation). Fractions (1 ml) were collected and proteins were separated by SDS-PAGE. Proteins were transferred to Immobilon P membranes for immunoblotting with monoclonal antibodies specific for Sec6, Sec8, or E-cadherin.

Immunofluorescent Staining of Sec6/8 Complex in MDCK Cells

Low-density, contact-naive, or confluent, polarized cultures of MDCK cells were fixed/permeabilized in 100% methanol at −20°C for 10 min. Where noted, cells were extracted with 1% Triton X-100 in phosphate-buffered saline (PBS) at 0°C for 10 min prior to methanol fixation. Nonspecific sites were blocked for 2 hr at 4°C in PBS containing 0.2% BSA and 1% goat serum. Primary antibodies against Sec6 (9H5 hybridoma supernatant diluted 1:4), Sec8 (8F12 ascites fluid diluted 1:100), ZO-1 (R40.76 ascites fluid diluted 1:200), or E-cadherin (UVO polyclonal antiserum diluted 1:500) were diluted in blocking buffer and applied to cells for 2 hr at 4°C. Following three washes in blocking buffer, fluorescein- or rhodamine-conjugated

secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:200 in blocking buffer, were applied for 1 hr at 4°C. Samples were washed three times in blocking buffer and mounted in VectaShield (Vector Laboratories, Burlingame, CA). Samples were viewed with either a Zeiss Axioplan microscope (63× objective) or a Molecular Dynamics laser confocal imaging system (Beckman Center Imaging Facility, Stanford University). For confocal images, x-z views were obtained by averaging sections over a line at each z position in 0.2 μm steps.

In Vitro Transport of $p75^{NTR}$ and LDLR in Permeabilized MDCK Cells

cDNAs encoding receptors for either nerve growth factor ($p75^{NTR}$) or low-density lipoprotein (LDLR) were introduced into polarized MDCK cells by adenovirus-mediated gene transfer. Replication-defective adenovirus vectors encoding $p75^{NTR}$ (AdCMVp75) and LDLR (AdRSVrLDLR) were kindly provided by Dr. Moses Chao and Dr. James Wilson, respectively. Recombinant adenovirus vectors were applied to the apical surface in serum-free DMEM for 2 hr. Following infection, DMEM/FBS was added, and cells were incubated at 37°C for 24 hr.

Cultures were washed twice with sulfate-free DMEM and incubated for 30 min at 37°C in the same medium. Filters were placed on 25 μl droplets of medium containing 1 mCi/ml $H_2{}^{35}SO_4$ (ICN) on a parafilm sheet; 200 μl of sulfate-free DMEM was added to the apical compartment. Cells were incubated at 19°C for 2 hr to accumulate newly synthesized proteins in the TGN (Matlin and Simons, 1983). Radiosulfate was incorporated into terminal carbohydrates during this incubation, allowing for specific labeling of $p75^{NTR}$ and LDLR in a late Golgi compartment.

Filters were rinsed twice with ice-cold KOAc(+) buffer (115 mM KOAc, 2.5 mM $Mg(OAc)_2$, 25 mM HEPES [pH 7.4], 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$) and once with transport buffer (20 mM HEPES-KOH [pH 8.0], 90 mM KOAc, 2 mM $Mg(OAc)_2$, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$). Steptolysin-O (purchased from Dr. Sucharit Bhakdi, Johannes Gutenberg University, Mainz, Germany) was diluted to 15 μg/ml in KOAc(−) buffer (115 mM KOAc, 2.5 mM $Mg(OAc)_2$, 25 mM HEPES, 2 mM DTT) immediately before use. For basal-lateral permeabilization of $p75^{NTR}$-expressing cells, filters were placed on 25 μl droplets of buffer containing SLO; for apical permeabilization of LDLR-expressing cells, 140 μl of buffer containing SLO was applied to the apical compartment. In both cases, 500 μl of transport buffer was applied to the opposite surface. Cells were incubated for 10 min on ice on a rocking platform. Filters were rinsed twice and washed for 5 min in transport buffer. To initiate permeabilization, cells were incubated for 45 min at 19°C in transport buffer containing protease inhibitors (1 mM PMSF and 10 μg/ml each of pepstatin A, leupeptin, and antipain). Transport buffer was changed, and filters were returned to 19°C for 30 min; where indicated, anti-Sec8 monoclonal antibodies (2E9, 2E12, 5C3, 10C2, 17A10, each diluted 1:100) were added to permeabilized surfaces. The effectiveness of SLO permeabilization was assessed by quantitating the release of LDH activity from SLO-permeabilized cells with an assay kit according to manufacturer's protocol (Sigma cat. #500, St. Louis, MO). Under conditions employed in transport assays, SLO permeabilization resulted in release of ~65% of the cytosolic LDH during the two incubation periods at 19°C. Furthermore, >90% of the cells were permeabilized, as monitored by the accessibility of an intracellular epitope to antibody present during the second 19°C incubation. The presence of functional tight junctions was verified by measuring the diffusion of ^{3}H-inulin across the monolayer.

Transport buffer containing an ATP-regenerating system (2 mM MgATP, 1 mM GTP, 16 mM creatine phosphate, and 0.16 mg/ml creatine kinase), 5 mg/ml bovine brain cytosol, protease inhibitors, and Sec8 antibodies, where indicated, was applied to the SLO-permeabilized surface of cells; 1 ml of transport buffer was applied to nonpermeabilized surfaces. As a control, one set of SLO-permeabilized filters was shifted to 0°C at this time to determine the amount of transport that occurred during incubation at 19°C. The remaining filters were incubated at 37°C for 1 hr, after which time all cultures were placed on ice and washed five times with Ringer's saline solution. Sulfo-NHS-SS-Biotin (Pierce, Rockford IL) (0.5 mg/ml in Ringer's saline) was applied to nonpermeabilized surfaces of cultures (0.2 ml apical/0.5 ml basal-lateral), and the cells were incubated twice for 20 min at 4°C with constant rocking; 0.5 ml of Ringer's saline was added to the opposite compartment. The biotinylation reaction was quenched by washing cells in five changes of Tris saline (120 mM NaCl, 10 mM Tris [pH 7.4]) containing 50 mM NH_4Cl and 0.2% BSA at 4°C. Cells were lysed for 1 hr in 1 ml/filter TX-100 lysis buffer. Lysates were centrifuged at 15,000 × g for 10 min, and supernatant fractions were transferred to clean tubes. Lysate (50 μl) was removed and mixed with SDS-PAGE sample buffer for quantitation of total protein expression. The remaining lysate (950 μl) was combined with 50 μl avidin-agarose (Pierce) and mixed overnight at 4°C. Precipitates were washed as described (Le Bivic et al., 1991), and biotinylated proteins were eluted by boiling samples in SDS-PAGE sample buffer. The amount of labeled protein was determined directly using a phosphorimager. Values reported for "percent transport" were calculated by dividing the amount of avidin-precipitated protein by the amount of total labeled protein in the sample.

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